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MD-2 Binds to Bacterial Lipopolysaccharide*

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ABSTRACT

The exact roles and abilities of the individual components of the lipopolysaccharide (LPS) receptor complex of proteins remain unclear. MD-2 is a molecule found in association with toll-like receptor 4. We produced recombinant human MD-2 to explore its LPS binding ability and role in the LPS receptor complex. MD-2 binds to highly purified rough LPS derived from *Salmonella minnesota* and *Escherichia coli* in five different assays; one assay yielded an apparent K_D of 65 nM. MD-2 binding to LPS did not require LPS-binding proteins LBP and CD14; in fact LBP competed with MD-2 for LPS. MD-2 enhanced the biological activity of LPS in toll-like receptor 4-transfected Chinese

hamster ovary cells but inhibited LPS activation of U373 astrocytoma cells and of monocytes in human whole blood. These data indicate that MD-2 is a genuine LPS-binding protein and strongly suggest that MD-2 could play a role in regulation of cellular activation by LPS depending on its local availability.

INTRODUCTION

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Mammals respond to microbial invasion with both innate and adaptive immune responses. The LPS[1] of Gram-negative bacteria is a potent initiator of the innate inflammatory response that has an important antimicrobial role. For that reason, and because Gram-negative bacteria are common initiators of septic shock, the receptors for LPS have received much scrutiny in recent years. Recent work has shown that myeloid cells use at least four proteins to mount a sensitive cellular response to LPS: LBP, CD14, TLR4, and MD-2 (1-4). CD14 is a critical receptor for LPS because anti-CD14 monoclonal antibody (Mab) can inhibit LPS-induced effects both *in vitro* and *in vivo* (5, 6). However, because CD14 has no transmembrane domain, it cannot transmit a transmembrane signal. The type 1 transmembrane protein TLR4 is probably the signal-transducing component of the LPS receptor, based on genetic studies and a variety of other studies (3, 7, 8). Comparative studies of TLR4 from mice and humans suggest that it interacts directly with LPS (9), as do cross-linking studies with a photoactivable derivative of LPS (10). MD-2 was identified as a TLR4-binding protein that is required for the function of TLR4 (4, 11). Like CD14, it has no transmembrane domain and apparently remains cell-associated because it binds to TLR4 (4). In this work, we describe the use of several biochemical techniques to address the question of whether LPS interacts directly with MD-2 in the absence of other LPS-binding proteins and investigate the role of this interaction in cellular activation by LPS.

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Reagents

Re595 LPS and its FITC and ^{125}I-sulfosuccinimidyl-2-(*p*-azidosalicylamido)-1,3-dithiopropionate derivatives (^{125}I-ASD-LPS) were prepared as described previously (12, 13). Labeling results obtained with this material were unchanged (10) by repurification according to Hirschfeld *et al.* (14). Biotinylated Re595 LPS (biotin-LPS) was a gift from R. Dziarski and had no TLR2 agonist activity.[2] Tritiated Ra LPS ([^{3}H]LPS) was obtained from List Biochemicals. Partially disaggregated LPS from *Escherichia coli* K-12 strain LCD25 was prepared as described previously (15). Recombinant soluble CD14 and LBP with C-terminal His tags were prepared as described previously (16). Control protein was a 62-kDa heat shock protein of *Coccidioides immitis* with a C-terminal His tag (a gift from A. Kanauchi). Polyclonal rabbit anti-MD-2 IgG was purified using a protein A column (Amersham-Pharmacia Biotech) from rabbit sera immunized with purified recombinant MD-2 by QED Biosciences Inc. Mab to MD-2, 5H10, was generated as described previously (17) using recombinant MD-2. The IgG1 fraction was purified from the culture supernatant

by protein G (Amersham Pharmacia Biotech) affinity chromatography. Mabs to TLR4, HTA 405, HTA 414, HTA 125, and HTA 1216 were generous gifts from K. Miyake. Control mouse IgG1 and rabbit IgG were obtained from Caltag. All protein biotinylations were done using the EZ-Link Sulfo-NHS-LC Biotinylation Kit (Pierce). All reagents were tested for LPS contamination with Limulus Amoebocyte Lysate (BioWhittaker). When necessary, LPS was removed from the reagents using END-X (Associates of Cape Cod, Inc.).

Cloning and Expression of MD-2

The human MD-2 cDNA clone (accession number AA099571) was obtained from Genome System Inc. The coding sequence, excluding the signal sequence, was amplified from plasmid DNA by polymerase chain reaction using Takara LA *ta*-DNA polymerase (Pan Vera). The fragments were cloned into baculovirus transfer vector pBAC11 (Novagen) for expression of secretory protein using the gp64 signal peptide sequence. The MD-2 that contains the C-terminal fusion 6His tag was made by cloning the polymerase chain reaction fragment into *NcoI*/*Nhe* sites. The use of this vector also allowed cleavage of the His tag from the expressed protein using enterokinase.

To generate recombinant baculovirus, the recombinant pBAC11 plasmids containing the desired inserts were co-transfected with Bacvector 2000 DNA (Novagen) into sf9 cells using Eufectin (Novagen) as described by the manufacturer's protocol. The recombinant viruses generated from the sf9 cells were plaque-purified three times by the plaque assay method.

The purified viruses were amplified to produce a high titer virus stock and used to infect Hi5 cells for protein production. The cells and supernatant were harvested 48-72 h after infection of the virus at multiplicity of infection > 5 by the National Cell Culture Center. The expressed protein was purified from the cell culture supernatant by nickel affinity chromatography as described elsewhere (16). The purified protein was analyzed on 10% or 4-12% Nu-PAGE gels with 2-(*N*-morpholino) ethane sulfonic acid buffer (Novex) and Western blotted onto polyvinylidene difluoride membrane. The His tag protein was identified with anti-penta-His antibody (Qiagen) and peroxidase-labeled sheep anti-mouse Ig using the ECL Western blotting system (Amersham Pharmacia Biotec.). The protein was >90% pure as judged by PAGE and gel filtration on high pressure liquid chromatography.

Removal of His Tag from MD-2

The His tag was removed from MD-2 by cleavage with enterokinase, which recognizes amino acids DDDK between the C-terminal of MD-2 and the His tag. 1 unit of EKMax (Invitrogen), the catalytic subunit of enterokinase, was added per 10 μg of protein in PBS, typically at 0.5 μg/ml protein. The reaction was allowed to proceed at 4 °C for 18 h. The EKMax was removed by incubation with 15 μl EK-Away (Invitrogen)/unit EKMax. Any uncleaved protein and free His tag were then removed in two incubations, each using 0.35 μl Ni-NTA Superflow (Qiagen)/starting μg protein. The cleaved product was assayed for the absence of His tag by Western blotting with Mab to His tag (Qiagen).

LPS Binding Assays

ELISA Assays-- Two binding ELISAs were performed, immobilizing LPS or MD-2. In the immobilizing MD-2 ELISA, a 96-well plate (Immulon2; Dynatech) was coated with MD-2, CD14, or BSA in 0.2 M acetate buffer, pH 5.0, at 25 °C. The concentration of MD-2 was 25 μg/ml; the concentration of CD14 and BSA was 50 μg/ml. The wells were blocked and washed as described previously (16). Biotin-LPS was added (16) at increasing concentrations and incubated for 30 min at 37 °C. In some samples, polymyxin B (10 μg/ml) was added to the biotin-LPS before addition to the wells. After further washing, streptavidin-alkaline phosphatase conjugate was added, and the color reaction was developed and measured as described previously (16).

The assay for MD-2 binding using immobilized LPS was done similarly to the method described previously (18). Re595 LPS complexed to BSA was used to coat a microtiter plate (Dynatech 2205). The plate was incubated for 3 h at 37 °C, rinsed with water, and air-dried overnight. Wells with no LPS were incubated overnight with 10 mg/ml BSA in 50 mM HEPES and 0.15 M NaCl, pH 7.4 (HS buffer). MD-2 protein with or without the His tag, diluted in 1 mg/ml BSA in HS buffer, was added in a total volume of 100 μl/well. After washing, MD-2 was detected by biotinylated monoclonal antibody 5H10 at 2.5 μg/ml diluted in 1 mg/ml BSA in HS buffer. Bound biotin-5H10 was detected using streptavidin-horseradish peroxidase (Zymed Laboratories Inc.) and its substrate, 1% tetramethylbenzidine in dimethyl sulfoxide in 0.1 M sodium acetate, pH 6.0, and 0.01% hydrogen peroxide (TMB). After the reaction was stopped with 1.2 M sulfuric acid, the absorbance of each well was measured at 450 nm. Inhibition of MD-2 binding was studied by mixing LPS with 2 μg/ml (100 nM) MD-2 for 3 h at 37 °C before adding the mixture to the plate. The calculation of MD-2 and LPS molarity was based on the assumption that the formular weight of LPS is 2,500, and the formular weight of MD-2 is 20,000.

The Fluorescence Assay-- Binding of FITC-LPS to MD-2 was done as described previously (16, 19). Briefly, 10 ng/ml FITC-LPS was mixed with MD-2 in an SLM fluorimeter, and the fluorescence intensity was measured as a function of time. The apparent K_D was estimated using a variety of concentrations of MD-2, measuring the increase in fluorescence signal at each concentration and analyzing the result graphically (Fig. 3).

Photoaffinity Labeling-- ^{125}I-ASD-LPS (0.5 μg/ml) was incubated with various concentrations of proteins in 25 mM HEPES and 2.5 mM EDTA, pH 7.4, at 37 °C for 10 min, followed by photolysis on ice for 5 min. The samples were analyzed on an SDS-polyacrylamide gel as described previously (12).

Sucrose Density Gradients-- The experiments were done by mixing [^{3}H]LPS with MD-2. The complexes were analyzed on a linear 5-20% sucrose gradient resting on a cushion of 40% sucrose as described previously (19). The gradients were centrifuged at 55,000 rpm in a VT865 rotor (Sorvall) for 80 min. 350-μl fractions were collected, and the amount of [^{3}H]LPS was determined by liquid scintillation counting.

Native PAGE-- Native PAGE was performed according to the previously published method (20). MD-2 (30 μg/ml, 1.5 mM) was incubated with 0-500 μg/ml (0-200 μM) Re595 LPS in PBS with 1 mM EDTA in a total volume of 10 μl at 37 °C for 30 min. The loading buffer was added, and the mixture was electrophoresed using 4-20% nondenaturing PAGE (Novex). The gel was then silver-stained using

silverXpress (Novex) according to the manufacturer's protocol.

Construction of TLR2 and TLR4 Expression Plasmids

The coding regions of TLR2 and TLR4 minus the N-terminal signal sequences were polymerase chain reaction-amplified using Takara LA *Taq* polymerase (Pan Vera) from plasmids containing TLR4 (gifts from Drs. P. Godowski and C. Janeway). The fragments were subcloned into the *Sac*II/*Sal*I sites of pDisplay (Invitrogen). The expressed protein contains a murine Ig k-chain leader sequence and an N-terminal hemagglutinin A (HA) epitope tag.

Cell Culture and Transfection

Cell lines were maintained in the laboratory as described previously (13, 16). The CHO cell line stably transfected with inducible membrane CD25 under the transcriptional control of a human E-selectin promotor containing NF-κB binding sites was a gift from Dr. Douglas Golenbock (21). Plasmid DNA was prepared using an Endo free kit (Qiagen). Stably transfected cell lines were generated using LipofectAMINE 2000 (Life Technologies, Inc.) according to the manufacturer's protocol. The cells expressing TLR proteins were sorted by immunomagnetic beads (Dynal) using anti-HA Mab (Roche Diagnostic Corp.). The stably transfected lines were generated by selection with G418 (Life Technologies, Inc.). Transfected cells were assayed for surface expression of the HA epitope by FACS analysis using anti-HA Mab followed by the $F(ab')_2$ fragment of goat anti-mouse Ig-FITC (Caltag).

Analysis of MD-2 Binding to TLR4

TLR4-transfected CHO cells with or without the CD25 reporter gene were incubated with 1 μg/ml MD-2 in RPMI 1640 medium with 10% fetal calf serum for 10 min at 20 °C. After washing off the excess protein with the medium, the cells were stained with anti-His Mab (Qiagen) to detect MD-2 or anti-HA to detect TLR4 followed by rabbit anti-mouse Ig-FITC and analyzed by FACS analysis as described previously (13).

Analysis of NF-κB Activity by Flow Cytometry

CHO cells carrying NF-κB reporter plasmids to express surface CD25 were diluted to 2.5×10^5 cells/ml in Dulbecco's modified Eagle's medium/F-12 supplemented with 10% fetal calf serum without antibiotics and plated in a 24-well plate at 0.5 ml/well 1 day before the activation. The cells were stimulated in 0.3 ml of fresh medium and incubated overnight. The cells were harvested using PBS and 1 mM EDTA, stained with phycoerythrin-CD25 Mab (Becton Dickinson) and analyzed by FACS analysis as described previously (13).

Assay for LPS Activation in THP-1 Cells

THP-1 cells were purchased from American Type Culture Collection and cultured in RPMI 1640 medium with 10% heat-inactivated fetal calf serum, 2 mM glutamine, 10 mM HEPES, and 50 μg/ml gentamycin. The cells were cultured with 100 nM 1_{α},25-dihydroxyvitamin D_3 (Calbiochem) for 72 h before activation. The cells were plated in 96-well tissue culture plates at 10^5 cells/well and

activated with LPS with or without 20 μg/ml various antibodies for 6 h. The supernatant was harvested and assayed for IL-8 production by ELISA. In the ELISA assay, IL-8 was captured with anti-human IL-8 antibody (R&D Systems) and detected by rabbit anti-IL-8 (Endogen) followed by goat anti-rabbit IgG-horseradish peroxidase (Tago) and the substrate TMB. The plate OD was read at 450 nm, and the amount of IL-8 was determined using a standard curve derived from purified IL-8 (Genzyme).

Activation of U373 Cells

The cells were cultured in a 96-well plate and activated with various reagents in minimal essential Earle's medium supplemented with 10 mg/ml human serum albumin. The supernatant was harvested after 16 h of activation and assayed for IL-6 by ELISA as described previously (16).

Whole Blood Assay

Peripheral venous blood from a normal volunteer was collected in heparin (10 units/ml), and recombinant human MD-2 or vehicle (PBS, 0.1 mg/ml BSA) was added to a final concentration of 10 μg MD-2/ml whole blood without significantly diluting the blood. The blood was transferred to 12 × 75-mm polypropylene tubes (Falcon 2063) (190 μl/tube), 10 μl of LPS (*E. coli* K-12 strain LCD25) was added, and the mixtures were incubated for 5 h at 37 °C in a 5% CO_2 atmosphere. The incubations were stopped by adding 600 μl of cold PBS, the cells were removed by centrifugation (10 min at 500 × *g*), and cytokines were measured in the supernatants by ELISA. Tumor necrosis factor α, IL-1β, and IL-6 were measured by ELISA using OptEIA ELISA set reagents (BD Pharmingen) according to the manufacturer's instructions.

RESULTS

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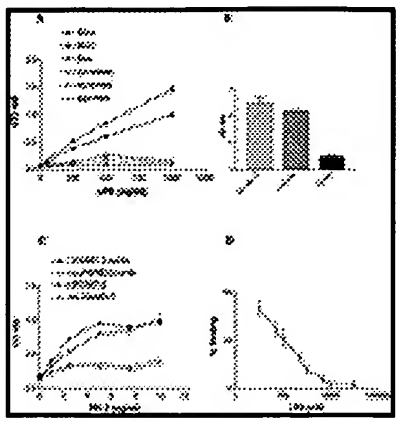
MD-2 Binds to LPS– The ability of MD-2, BSA, and CD14 to bind biotin-LPS was first tested in two ELISA formats (Fig. 1). Fig. 1*A* shows that, when immobilized on a plate, MD-2 and CD14 bind much more LPS-biotin than does BSA. The binding of MD-2 and CD14 is LPS dose-dependent. If the biotin-LPS is preincubated with polymyxin B, binding to MD-2 and CD14 is inhibited. This suggests that MD-2 is binding to the lipid A region of LPS. Fig. 1*B* shows that when the protein is preincubated with polyclonal rabbit anti-MD-2, the binding can be inhibited. MD-2 binding to LPS could also be demonstrated when LPS was immobilized on the plate (Fig. 1*C*). The protein without His tag binds to LPS as well as the protein with His tag, and minimal binding of MD-2 occurred when there was no LPS on the plate. Thus, His tag at the C terminus of the protein is not involved in LPS binding. Fig. 1*D* shows that the binding of MD-2 to immobilized LPS can be inhibited by preincubating MD-2 with LPS in a dose-dependent manner. This suggests that the binding is specific. The LPS concentration necessary to reduce the binding of 100 nM MD-2 to the plate by 50% is about 100 nM, suggesting a one-to-one molecular stoichiometry in the MD-2·LPS complex and an apparent K_D near 100 nM.

Fig. 1. LPS protein binding in ELISA formats. *A*, the



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ELISA plate was coated with MD-2, CD14, or BSA. Increasing concentrations of biotin-LPS or biotin-LPS with polymyxin B (*PMB*) were added, and the amount of bound LPS was detected with streptavidin-alkaline phosphatase. *B,* ELISA assay for biotin-LPS binding to an MD-2-coated plate in the presence of control rabbit IgG or purified rabbit anti-MD-2 IgG. *C,* ELISA assay for protein binding to an LPS-coated plate. The plate was coated with LPS or buffer (*noLPS*), and increasing concentrations of MD-2 or MD-2 without a His tag (*MD2-noHis*) were added to the plate. MD-2 binding to the plate was detected using biotinylated anti-MD2 Mab 5H10. *D,* inhibition of MD-2 binding to an LPS-coated plate. MD-2 at 2 μg/ml (100 nM) was preincubated for 3 h at 37 °C in the absence or presence of various concentrations of LPS before being added to the plate. MD-2 binding to the plate was detected as described in *C,* and the results from three duplicate experiments are presented as a percentage of binding of MD-2 in the absence of inhibitor.

MD-2 also binds to LPS in the fluid phase, as shown in four different assays. Re595 LPS derivatized with a radioactive, photoactivable group (^{125}I-ASD-LPS) can be used to detect LPS-binding proteins by the covalent attachment of the radiolabeled group to the protein upon photolysis. Previous work has shown the remarkable selectivity of this reagent (12, 22). Fig. 2 shows the results of a photoaffinity labeling experiment. Fig. 2*A* shows that LBP and CD14 were strongly labeled by ^{125}I-ASD-LPS, whereas the 62-kDa control His tag protein shows no labeling. Labeling of MD-2 is shown in Fig. 2*B*. The *far left lane* shows MD-2 interaction with ^{125}I-ASD-LPS in the absence of LBP and CD14. The figure shows that between 0.3 and 1 μg of LBP effectively competes with 3 μg of MD-2 for the available ^{125}I-ASD-LPS, whereas 3 μg of CD14 does not compete effectively. The reported apparent K_D for LPS binding to LBP is 3.5×10^{-9} M, and the reported apparent K_D for LPS binding to CD14 is between 30 and 74×10^{-9} M (16, 19).

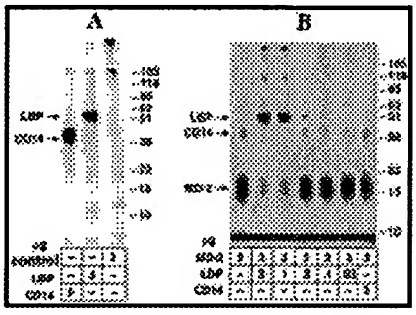

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Fig. 2. Photoaffinity cross-linking of LPS to LPS-binding proteins. Protein as indicated in the figure was incubated with 0.5 μg/ml ^{125}I-ASD-LPS for 10 min at 37 °C in 50 μl of buffer and photolysed. The samples were analyzed by SDS-PAGE and autoradiography. The mobilities of LBP, CD14, and MD-2 are shown at the *left*; molecular mass standards are shown at the *right. A,* labeling of LBP, CD14, and a 62-kDa His tag protein (*control*). *B,* labeling of MD-2 in the presence of LBP or CD14.

When fluoresceinated derivatives of LPS (FITC-LPS) are bound by LBP or CD14, an increase in the

fluorescence signal is observed (16, 19). We found that MD-2 similarly enhanced the fluorescence of FITC-LPS (Fig. 3*A*), although not to as great an extent as LBP or CD14 has in previously reported experiments (16, 19). Analyzing the degree of fluorescence enhancement as a function of MD-2 concentration (Fig. 3*B*), an apparent K_D of 65 nM was obtained. This is similar to the measured affinities of FITC-LPS for CD14 (16, 19).

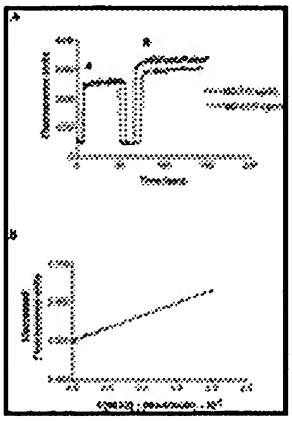

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Fig. 3. Interaction of MD-2 with FITC-LPS. *A,* the fluorescence intensity of FITC-LPS was measured using an SLM fluorimeter before and after the addition of MD-2. FITC-LPS was added at 10 s (*A*), and MD-2 (1 or 10 μg/ml) was added at 75 and 65 s (*B*), respectively. The drop in the trace to baseline is caused by the closing of shutters during reagent addition. *B,* double reciprocal plot to determine the apparent K_D for MD-2 binding to LPS; K_D = slope/*y* – intercept = 65 nM.

We also used sucrose density gradients to analyze the state of LPS upon binding to MD-2. In previously published experiments (16, 19), we have found that LBP and CD14 caused a redistribution of [^{3}H]LPS in the gradients such as those shown in the experiments here. Fig. 4 shows the sedimentation measurements of two concentrations of [^{3}H]LPS in the absence and presence of two concentrations of MD-2. In the absence of MD-2 (Fig. 4, *A* and *D*), most of the LPS (83-88%) sediments near the bottom of the tube, presumably due to its highly aggregated state, leaving only a small amount (12-17%) in fractions 8-13 near the top of the tube. In the presence of MD-2 at either concentration (Fig. 4, *B, C, E,* and *F*), the amount of [^{3}H]LPS in fractions 8-13 increases ~3-fold. In experiments not shown, fractions 8-13 are where MD-2 was found in these gradients.

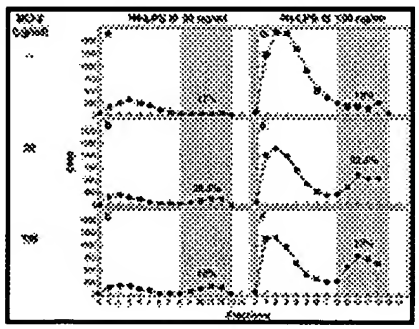

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Fig. 4. Sucrose density gradient analysis of [^{3}H] LPS·MD-2 complexes. Mixtures of [^{3}H]LPS (30 or 130 ng/ml) and MD-2 (0, 20, or 100 μg/ml) were analyzed on sucrose density gradients. Fractions were assayed for [^{3}H] LPS by liquid scintillation counting. Fraction 0 was obtained by washing the emptied tube with 1% SDS to look for tube-absorbed [^{3}H]LPS. Fraction 1 corresponds to the bottom of the tube. The fraction of counts recovered in fractions 8-13 is indicated.

Yet another means we have used to assess the interaction between LPS and a protein is native PAGE. It was previously shown that the binding of strong negatively charged LPS to sCD14 caused a characteristic increase in the mobility of the protein on native gels (20). Fig. 5 shows that at 1.5 μM MD-2, most of the MD-2 changes mobility in the presence of 2 μM LPS. This indicates that the molar ratio of LPS binding to MD-2 is 1:1 and is in agreement with the result obtained by our ELISA competition assay (Fig. 1*D*). The MD-2 mobility continues to increase with increasing LPS, possibly due to the binding of multiple LPS molecules. This also occurred with sCD14 (20). The figure also shows that all of the protein changes mobility, which indicates that all MD-2 binds to LPS rather than just some denatured portion binding LPS nonspecifically.

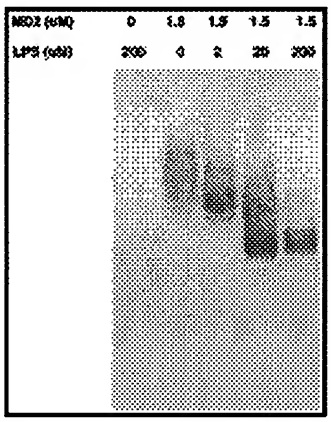

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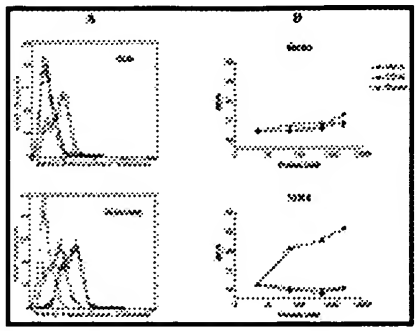
Fig. 5. Native PAGE analysis of MD-2·LPS complexes. MD-2 (1.5 μM, 30 μg/ml) was incubated in the absence and presence of increasing LPS concentrations at 37 °C for 10 min. A shift in the electrophoretic mobility of MD-2 was detected by silver staining after running the mixture on a 4-20% native PAGE.

The results of the ELISA, photoaffinity labeling, FITC-LPS, sucrose density gradient, and native PAGE experiments lead to the conclusion that MD-2 is a genuine LPS-binding protein.

MD-2 Binds to TLR4— In previously reported studies, Shimazu *et al.* (4) have shown that MD-2 associates with TLR4 by co-transfection of MD-2 and TLR4 genes into BaF/3 cells. Here we show that MD-2 binds to TLR4-transfected CHO cells simply by adding the protein to the cells. Fig. 6*A* shows that the cells expressed TLR4 as detected by anti-HA, but no MD-2 was detectable with the anti-His tag. When MD-2 was added, the cells became brightly stained with anti-His, whereas anti-HA staining remained the same (Fig. 6*A, bottom panel*). This suggests that MD-2 bound to TLR4-transfected CHO cells. The same result was obtained using TLR4-transfected CHO cells carrying the CD25 reporter plasmid (data not shown). No binding of MD-2 was observed to cells transfected with TLR2, CD14, or vector alone (data not shown).

Fig. 6. MD-2 binds to TLR4-transfected CHO cells and mediates TLR4 activation by LPS. *A,* flow cytometry



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analysis of TLR4-transfected CHO cells. The *top panel* shows TLR4-transfected CHO cells, and the *bottom panel* shows TLR4-transfected CHO cells after incubation with 1 μg/ml MD-2. The *dashed line* shows background staining with second antibody alone. The *thin black line* shows the fluorescence intensity of anti-HA staining for TLR4 expression. The *thick black line* shows the intensity of anti-His staining to detect MD-2. *B*, TLR4- and vector (pDisplay)-transfected CHO cells harboring CD25 reporter plasmids were activated with 100 ng/ml LPS in the presence of increasing concentrations of MD-2, CD14, and control His tag protein. The NF-κB activation was measured as the mean channel number obtained after FACS analysis of CD25 surface expression using anti-CD25-phycoerythrin.

LPS Activation through TLR4 Is Dependent upon MD-2-- We tested the requirement for MD-2 on LPS activation of a TLR4-transfected CHO cell line. This CHO cell line is stably transfected with an NF-κB-driven CD25 gene so that surface CD25 can be measured by FACS analysis as an indication of NF-κB activation (21). Fig. 6*B* shows the FACS analysis of CD25 expression as the mean channel number of fluorescence intensity in TLR4- and vector only-transfected cells 16 h after the addition of 100 ng/ml Re595 LPS in the presence of MD-2, CD14, or control His tag protein. The expression of CD25 increased in a dose-dependent manner only in TLR4-transfected cells and only when MD-2 was present. CD14 or control protein had no effect on LPS activation through TLR4. When MD-2 with no His tag was used, an identical result was obtained (data not shown).

We also investigated the role of MD-2 in LPS activation of the human macrophage-like cell line THP-1. The cells became LPS-sensitive after induction of CD14 with 1_{α},25-dihydroxyvitamin D_3 (22). THP-1 cells express TLR4 and MD-2 as detected by FACS analysis using anti-TLR4 Mabs and rabbit anti-MD-2 (data not shown). Fig. 7 shows that the response of 1_{α},25-dihydroxyvitamin D_3-treated THP-1 cells to Re595 LPS, as measured by an IL-8 assay, can be inhibited by polyclonal rabbit anti-MD-2 as well as by anti-TLR4, *i.e.* the HTA series, and anti-CD14, 28C5. Our results indicate that not only TLR4 and CD14, as reported previously (23), but also MD-2 play roles in LPS activation in THP-1 cells. Because rabbit anti-MD-2 inhibits LPS binding to MD-2 (Fig. 1*B*), these results suggest that MD-2 must bind to LPS for cellular activation.

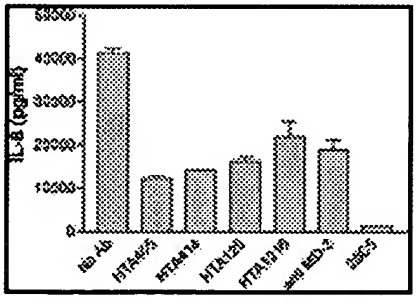

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Fig. 7. Antibody inhibition of LPS activation in THP-1 cells. An IL-8 response was measured by ELISA 6 h after 1 α,25-dihydroxyvitamin D_3-treated THP-1 cells were activated with 10 μg/ml LPS in the presence or absence of antibodies at 10 μg/ml. Antibodies are Mab anti-TLR4 (HTA series), rabbit polyclonal antibody anti-MD-2, and Mab anti-CD14 (28C5).

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MD-2 Inhibits LPS Response in U373 Cells– In contrast to the CHO-TLR4 cells that express a high level of TLR4, we observed an inhibitory effect of MD-2 upon CD14-dependent LPS activation in cells that express very low levels of surface TLR4. The U373 cells possess mRNA for TLR4 and MD-2 but not for TLR2 as observed by reverse transcription-polymerase chain reaction (data not shown). Surface expression of TLR4 or MD-2 cannot be detected by staining with antibodies to TLR4 or MD-2 (data not shown). LPS activates U373 cells in the presence of CD14 to produce IL-6 (16). Fig. 8*A* shows that MD-2 inhibits IL-6 secretion by the U373 cells activated with CD14 and LPS in a dose-dependent manner. At 100 ng/ml Re595 LPS and 1 μg/ml CD14, >40 ng/ml IL-6 was secreted from the U373 cells. When MD-2 was added from 1-10 μg/ml, the IL-6 response was inhibited by about 30-80%, respectively. Therefore, at a MD-2:CD14 molar ratio of as little as 3:1 (1 μg/ml of MD-2), LPS can be neutralized. In the photoaffinity labeling experiment (Fig. 2*B, far right column*), we show that LPS associates mostly with MD-2 at this molar ratio of MD-2:CD14. This suggests that MD-2 may inhibit sCD14-dependent activation of U373 cells by competing with sCD14 for binding to LPS.

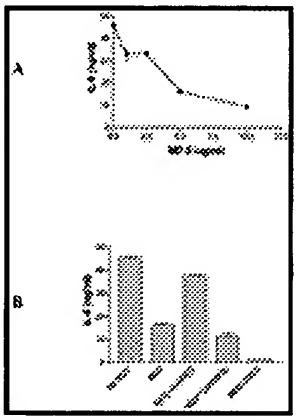

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Fig. 8. Effect of MD-2 on CD14-dependent LPS activation in U373 cells. *A,* IL-6 response in U373 cells after activation with 10 ng/ml LPS and 1 μg/ml CD14 in the presence of an increasing concentration of MD-2. *B,* IL-6 response in U373 cells after activation with 10 ng/ml LPS and 1 μg/ml CD14 in the presence or absence of MD-2 and in the presence of MD-2 with the addition of antibodies at 10 μg/ml as indicated. The effects of added rabbit anti-MD-2, control rabbit IgG, and Mab anti-CD14 (28C5) are shown.

We tested whether MD-2 binding to LPS contributes to its inhibitory role in LPS activation of U373 cells by using an antibody to MD-2 that inhibits MD-2 binding to LPS to reverse this effect. Fig. 8*B* shows that the IL-6 response in U373 cells in the presence of sCD14 and LPS was blocked by adding MD-2. Rabbit anti-MD2 significantly restored the response, suggesting that the exogenous MD-2 is sequestering LPS. As expected, Mab to CD14 inhibited the IL-6 response very efficiently.

MD-2 Inhibits LPS Activation in Whole Blood– The effect of MD-2 on LPS-induced activation in heparinized whole blood was determined. We activated whole blood with a variety of LPS concentrations in the presence or absence of 10 μg/ml MD-2. We measured tumor necrosis factor α, IL-1 β, and IL-6 production in response to LPS. As shown in Fig. 9, MD-2 at 10 μg/ml significantly decreased

tumor necrosis factor α, IL-1β, and IL-6 release from whole blood stimulated with LPS in concentrations ranging from 10 pg/ml to 10 ng/ml. The same concentration of MD-2 did not inhibit the ability of recombinant human IL-1β to stimulate IL-6 in whole blood (data not shown).

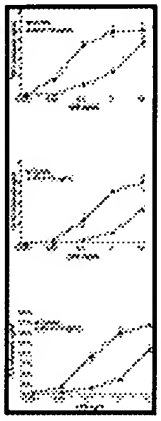

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Fig. 9. Effect of MD-2 on LPS dose response in heparinized whole blood. Whole human blood was activated with increasing concentrations of LPS in the presence or absence of 10 μg/ml MD-2. The release of tumor necrosis factor α, IL-1β, and IL-6, as measured by ELISA, is shown in the *top, middle,* and *bottom panel,* respectively.

DISCUSSION

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Our principal finding is that LPS will bind to MD-2 without assistance from either LBP or CD14. Whereas most of the experiments were done with His-tagged MD-2, selected experiments were done with MD-2 from which the His tag had been removed, so that here, as we have shown previously for CD14 (16), the His tag is irrelevant to the binding of LPS. In some of the assays, we also studied whether a His-tagged 62-kDa heat shock protein from *C. immitis* would bind LPS with negative results. Previous data (10) using MD-2 associated with TLR4 expressed on HEK293 cells suggested that CD14, TLR4, and MD-2 needed to be simultaneously present for efficient labeling of MD-2 (or TLR4) by ^{125}I-ASD-LPS. In those experiments (10), only cell-associated components were examined for labeling. Direct labeling of MD-2 would not have been observed because MD-2 would not have been cell-associated in the absence of TLR4 (4). The affinity of MD-2 for LPS appears to be near that of CD14, with an apparent K_D of 65 nM as shown by direct measurement using FITC-LPS. The sucrose density gradient experiments show that LPS binding to MD-2 is accompanied by LPS disaggregation. The FITC-LPS experiments are also consistent with this interpretation. The molar ratio of LPS:MD-2 appears to be 1:1 as shown by ELISA and native PAGE assays.

Our experiments with the binding of MD-2 to CHO cells expressing TLR4 suggest that MD-2 can associate with TLR4 without any need for co-expression of the two molecules. As far as we are aware, all previous studies with MD-2 have used MD-2 expression in the cells under study as the source of the

MD-2 (4, 11).

The functional effects of exogenously added MD-2 were quite varied. In the TLR4-transfected CHO cells, the addition of MD-2 enhanced responses to LPS, whereas in two other systems (U373 cells and whole blood), the addition of MD-2 inhibited responses to LPS. This could be related to the levels of expression of TLR4 and the level of endogenously available MD-2. If the transfected CHO cells do not make enough endogenous MD-2 to partner with the overexpressed TLR4, then added MD-2 might enable that TLR4 to become active and the cells to show enhanced responses to LPS. With the U373 cells and monocytes in blood, any activation is occurring only through endogenous TLR4, and it is reasonable to guess that the cells are making sufficient MD-2 to partner with their own TLR4. In this case, as we observe, added MD-2 might be expected to compete for the available LPS and inhibit cellular activation. An alternative explanation is based on species specificity. This explanation recognizes that the transfected CHO cells derived from Chinese hamsters are expressing endogenous hamster TLR4, endogenous hamster MD-2, and transfected human TLR4, whereas the U373 cells and the whole blood used were derived from humans and, finally, that the MD-2 used is human. Kawasaki *et al.* (24) have looked at responses to LPS in all four combinations of mouse TLR4, mouse MD-2, human TLR4, and human MD-2. Their data show quite clearly that the TLR4 and MD-2 need to be from the same species to be functional in enabling an LPS-driven response. Thus, because the transfected CHO cells in our experiments making human TLR4 cannot also supply human MD-2, they may require added human MD-2 for function of the human TLR4. This hypothesis is under investigation.

Quite irrespective of these considerations about the varied effects of added recombinant MD-2 on the LPS-dependent responses of various systems, our results show that MD-2 can bind LPS in isolation and that the MD-2 used has biological activity, with the latter being manifested differently in different assay systems.

However, we were surprised to see a strong inhibitory effect of MD-2 on LPS responses in human whole blood. The inhibition appears to be specific for LPS because the response to IL-1β was not affected. It is possible that MD-2 outcompetes TLR4-bound MD-2 for LPS binding. These data suggest that MD-2 can regulate LPS responses in whole blood in a manner similar to high levels of sCD14. Although sCD14 can enhance cell responses to LPS, it can also inhibit LPS responses in whole blood and in cells that express membrane CD14 when the sCD14 concentration is at or above levels found in normal plasma (25). sCD14 can also promote the release of LPS that has bound to the monocyte surface (26). Our preliminary data (data not shown) suggest that MD-2 also promotes the release of LPS from monocyte surfaces. In normal human serum, MD-2 exists at the ng/ml level,[3] whereas in this experiment, we used MD-2 at 10 μg/ml. It is therefore unclear whether natural levels of MD-2 could play a role in regulating the LPS response *in vivo* unless there are circumstances in which its concentration rises dramatically. However, the ability of MD-2 to inhibit LPS activation in human whole blood suggests that it may be useful as a therapeutic for LPS toxicity *in vivo*.

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FOOTNOTES

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[2] R. Dziarski, personal communication.

[3] S. Viriyakosol, P. S. Tobias, R. L. Kitchens, and T. N. Kirkland, unpublished data.

ABBREVIATIONS

The abbreviations used are: LPS, lipopolysaccharide; BSA, bovine serum albumin; TLR, toll-like receptor; sCD14, recombinant soluble CD14; ^{125}I-ASD-LPS, radioiodinated sulfosuccinimidyl-2-(*p*-azidosalicylamido)-1,3-dithiopropionate derivative of *Salmonella minnesota* Re595 lipopolysaccharide; FITC, fluorescein isothiocyanate; PBS, phosphate-buffered saline; ELISA, enzyme-linked immunosorbent assay; CHO, Chinese hamster ovary; Mab, monoclonal antibody; biotin-LPS, biotinylated Re595 LPS; PAGE, polyacrylamide gel electrophoresis; HA, hemagglutinin A; NF-κB, nuclear factor κB; FACS, fluorescence-activated cell-sorting; IL, interleukin.

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1. Schumann, R. R., Leong, S. R., Flaggs, G. W., Gray, P. W., Wright, S. D., Mathison, J. C., Tobias, P. S., and Ulevitch, R. J. (1990) *Science* **249**, 1429-1431[Medline] [Order article via Infotrieve]
2. Wright, S. D., Ramos, R. A., Tobias, P. S., Ulevitch, R. J., and Mathison, J. C. (1990) *Science* **249**, 1431-1433[Medline] [Order article via Infotrieve]
3. Poltorak, A., He, X., Smirnova, I., Liu, M. Y., Huffel, C. V., Du, X., Birdwell, D., Alejos, E., Silva, M., Galanos, C., Freudenberg, M., Ricciardi-Castagnoli, P., Layton, B., and Beutler, B. (1998) *Science* **282**, 2085-2088[Abstract/Free Full Text]

4. Shimazu, R., Akashi, S., Ogata, H., Nagai, Y., Fukudome, K., Miyake, K., and Kimoto, M. (1999) *J. Exp. Med.* **189**, 1777-1782[Abstract/Free Full Text]
5. Ulevitch, R. J., and Tobias, P. S. (1995) *Annu. Rev. Immunol.* **13**, 437-457[CrossRef][Medline] [Order article via Infotrieve]
6. Leturcq, D. J., Moriarty, A. M., Talbott, G., Winn, R. K., Martin, T. R., and Ulevitch, R. J. (1996) *J. Clin. Invest.* **98**, 1533-1538[Abstract/Free Full Text]
7. Heine, H., Kirschning, C. J., Lien, E., Monks, B. G., Rothe, M., and Golenbock, D. T. (1999) *J. Immunol.* **162**, 6971-6975[Abstract/Free Full Text]
8. Lien, E., Means, T. K., Heine, H., Yoshimura, A., Kusumoto, S., Fukase, K., Fenton, M. J., Oikawa, M., Qureshi, N., Monks, B., Finberg, R. W., Ingalls, R. R., and Golenbock, D. T. (2000) *J. Clin. Invest.* **105**, 497-504[Abstract/Free Full Text]
9. Poltorak, A., Ricciardi-Castagnoli, P., Citterio, S., and Beutler, B. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97**, 2163-2167[Abstract/Free Full Text]
10. da silva Correia, J., Soldau, K., Christen, U., Tobias, P. S., and Ulevitch, R. J. (2001) *J. Biol. Chem.* **276**, 21129-21135[Abstract/Free Full Text]
11. Akashi, S., Shimazu, R., Ogata, H., Nagai, Y., Takeda, K., Kimoto, M., and Miyake, K. (2000) *J. Immunol.* **164**, 3471-3475[Abstract/Free Full Text]
12. Kirkland, T. N., Virca, G. D., Kuus-Reichel, T., Multer, F. K., Kim, S. Y., Ulevitch, R. J., and Tobias, P. S. (1990) *J. Biol. Chem.* **265**, 9520-9525[Abstract/Free Full Text]
13. Viriyakosol, S., and Kirkland, T. N. (1995) *J. Biol. Chem.* **270**, 361-368[Abstract/Free Full Text]
14. Hirschfeld, M., Ma, Y., Weis, J. H., Vogel, S. N., and Weis, J. J. (2000) *J. Immunol.* **165**, 618-622 [Abstract/Free Full Text]
15. Kitchens, R. L., and Munford, R. S. (1998) *J. Immunol.* **160**, 1920-1928[Abstract/Free Full Text]
16. Viriyakosol, S., Mathison, J. C., Tobias, P. S., and Kirkland, T. N. (2000) *J. Biol. Chem.* **275**, 3144-3149[Abstract/Free Full Text]
17. Stanley, H. A., and Reese, R. T. (1985) *Proc. Natl. Acad. Sci. U. S. A.* **82**, 6272-6275[Abstract]
18. Tobias, P. S., Soldau, K., and Ulevitch, R. J. (1989) *J. Biol. Chem.* **264**, 10867-10871 [Abstract/Free Full Text]
19. Tobias, P. S., Soldau, K., Gegner, J. A., Mintz, D., and Ulevitch, R. J. (1995) *J. Biol. Chem.* **270**, 10482-10488[Abstract/Free Full Text]
20. Hailman, E., Lichenstein, H. S., Wurfel, M. M., Miller, D. S., Johnson, D. A., Kelley, M., Busse, L. A., Zukowski, M. M., and Wright, S. D. (1994) *J. Exp. Med.* **179**, 269-277[Abstract]
21. Delude, R. L., Yoshimura, A., Ingalls, R. R., and Golenbock, D. T. (1998) *J. Immunol.* **161**, 3001-3009[Abstract/Free Full Text]
22. Tobias, P. S., Soldau, K., Kline, L., Lee, J. D., Kato, K., Martin, T. P., and Ulevitch, R. J. (1993) *J. Immunol.* **150**, 3011-3021[Abstract/Free Full Text]
23. Tapping, R. I., Akashi, S., Miyake, K., Godowski, P. J., and Tobias, P. S. (2000) *J. Immunol.* **165**, 5780-5787[Abstract/Free Full Text]
24. Kawasaki, K., Akashi, S., Shimazu, R., Yoshida, T., Miyake, K., and Nishijima, M. (2000) *J. Biol. Chem.* **275**, 2251-2254[Abstract/Free Full Text]
25. Haziot, A., Rong, G. W., Bazil, V., Silver, J., and Goyert, S. M. (1994) *J. Immunol.* **152**, 5868-5876 [Abstract/Free Full Text]
26. Kitchens, R. L., Wolfbauer, G., Albers, J. J., and Munford, R. S. (1999) *J. Biol. Chem.* **274**, 34116-34122[Abstract/Free Full Text]

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